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RECEPTOR-IONOPHORE INTERACTION

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The overall objective of these investigations was to develop the procedures for precise quantitation and molecular definition of glutamate-sensitive binding sites and ion channels in the mammalian brain, and to apply this technology to the examination of the synthesis and expression of glutamate-sensitive sites in neurons maintained in culture. These investigations were based on the refinement of technology developed in our laboratories for the isolation of a glutamate binding protein (GBP) from mammalian brain synaptic membranes. This protein represents a component of the macromolecular complex of glutamate-activated membrane receptors.

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The studies that we have completed indicate that the protein that we had initially identified as the GBP was a fragment of a larger size protein in synaptic membranes. This larger protein had a molecular weight of 71,000 and was identified through antibodies that we had raised to the smaller 14 kDa fragment. New procedures were developed for the isolation and characterization of synaptic membrane GBP without damage by proteolysis. These were successful and the intact protein of 71,000 molecular weight was isolated and identified as a GBP. The ligand binding characteristics of this GBP were indicative of a glutamate/quisqualate receptor. Polyclonal antibodies were raised against this newly purified, intact GBP and the antibodies were shown to label a 71 kDa protein in synaptic membranes.

Procedures were also developed and tested for the immunoaffinity extraction of newly synthesized GBP from extracted brain messenger RNA. In addition, procedures were developed for primary neuronal cultures from brain tissue of newborn animals and for labelling of surface proteins by immunohistochemical procedures. The antibodies were also used to label nerve cell processes and nerve cell body regions in intact brain tissue by means of immuno-gold histochemical procedures with silver enhancement.

Because of the advances in our ability to reconstitute this 71 kDa GBP and related membrane proteins in lipid membranes, in the last period of our work we concentrated in the reconstitution studies of receptor-ion channel function. These studies were met with marked success in reconstituted glutamate-activated sodium-ion flux through the liposome membranes and in development of patch clamp techniques for the measurement of opening and closing of single ion channel events. Finally, once success with patch clamp measurements was achieved we proceeded to develop lipid bilayer procedures for reconstitution of the glutamate receptor-ion channel proteins.

FINAL REPORT

The major goals of our investigations was to measure the levels and activity of the glutamate binding protein (GBP) in brain preparations and in neuronal tissue cultures and to refine our techniques for immunochemical detection of this protein and for its reconstitution and measurement of ion channel activity. We initiated the studies by first developing monoclonal and polyclonal antibodies against the GBP that we had purified from brain synaptic membranes. This protein had the characteristics of a glutamate recognition site for the glutamate/quisqualate type receptors. The antibodies developed against a 15,000 molecular weight protein exhibited highly specific reaction with synaptic membranes isolated from rat brain tissue and high specific reactivity with the isolated protein. However, when these antibodies were used in electrotransfer immunoblot experiments it was apparent that the protein that was being recognized in synaptic membranes was a 71,000 rather than a 15,000 molecular weight protein. These observations led us to reexamine the nature of the GBP in intact synaptic membranes. Our hypothesis was that the protein we had identified was probably a proteolytic fragment of a larger molecular weight protein in synaptic membranes.

A new procedure was developed for the isolation of the GBP in the presence of very high concentrations of inhibitors of proteases. This procedure involved three steps, the first being the processing of solubilized synaptic membrane extracts through glutamate affinity chromatography and elution of the specifically bound proteins by means of sodium azide. Sodium-azide was selected as the eluting medium because this agent inhibits glutamate binding to the previously isolated GBP. The second involved ion-exchange chromatography on DEAE sephadex. The third step of the procedure involved HPLC on molecular size exclusion columns. Processing of solubilized synaptic membrane preparations through these three steps of chromatography led to the isolation and very high enrichment of a 71,000 molecular weight protein which had glutamate-binding activity indicative of one glutamate-binding site per one 71,000 molecular weight protein. The specificity of the recognition site for other glutamate analogs was strongly suggestive that this protein recognized analogs that are known to interact with the quisqualate type of receptors but not the N-methyl-D-aspartate (NMDA) or the kainic acid receptors. The 71,000 molecular weight protein just as the 14,000 molecular weight protein previously isolated, was a glycoprotein. Finally antibodies raised against this 71,000 kDa labelled a 71,000 molecular weight protein band in synaptic membranes.

The results of these studies were published and we focussed our attention on the use of the antibodies that we had developed against this 71,000 molecular weight protein. First procedures were developed for immunohistochemical labelling of nerve cells in brain tissue and also for labelling of the immunoreactive proteins in nerve cells grown in primary tissue cultures. In order to achieve these goals we developed immuno-gold affinity labelling procedures that showed very selective labelling of neurons in intact brain tissue slices. The sensitivity of the immunogold procedure was markedly increased by using the procedure of silver enhanced labeling of the gold particles. These studies were performed at both the light and electron-microscopic levels and it became clear that the antibodies we had raised were labeling dendrites of nerve cells in regions of the brain where glutamate nerve pathways are known to exist, such as the hippocampus and cerebellum. In addition, under electron-microscopic examination it became very clear that approximately 80% of all labelling was occurring at the plasma membrane level, and in particular, in plasma membranes

associated with the synaptic region.

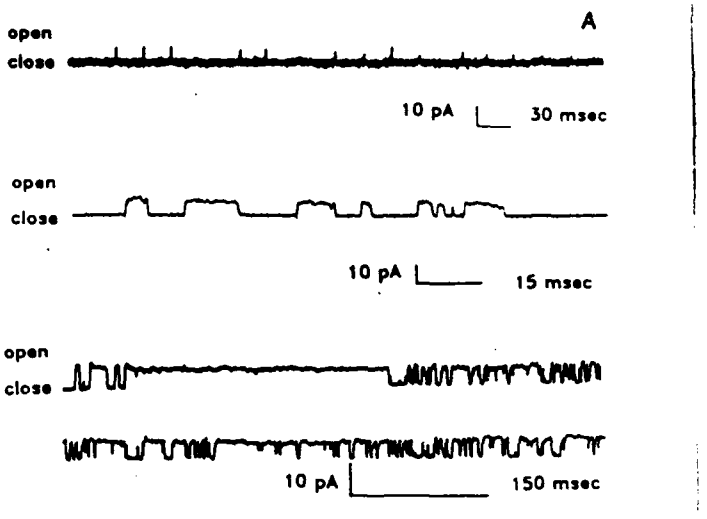
In order to accomplish the goal of studying the expression of this GBP in neurons, we developed the methodology of primary neuronal cultures from brain that we have found to have a high degree of immune-labelling by antibodies. Primary neuronal cultures in serum-free media were developed from the hippocampus and cerebellum and from the cerebral cortex of newborn rats. These primary neuronal cultures were characterized by determining the glial and neuronal cell population densities and than subsequently were examined for the presence of sites that could be labelled by the antibodies raised against GBP. The labelling that we observed is associated with surface proteins primarily and it is localized in discrete areas or "hot spots". These areas are most frequently in neuritic processes, possibly indicative of sites of synaptic contact by other neurons. The studies reporting the characterization of the antibodies raised against the 71,000 molecular weight protein as well as the immunohistochemical labelling described above are now being prepared for submission for publication. The studies with the primary neuronal cultures that were described in this paragraph are still ongoing and they represent the basis on which future studies of the expression of this protein in isolated primary neuronal cultures will be examined.

We plan to use the techniques of primary neuronal culture immunoreactivity in order to examine different aspects of the expression of GBP or glutamate receptors. In one group of studies we have already initiated an examination of glutamate-induced ion channel responses in these primary neuronal cultures. In the second series of studies we have initiated immuno-affinity extraction of newly synthesized proteins recognized by the antibodies. The latter group of studies are conducted by means of protein labelling with [^{35}S] methionine and immuno-affinity extraction of the proteins by means of the antibodies raised against the 71,000 molecular weight protein. These studies are still ongoing and only preliminary data have been obtained thus far.

Much of our attention more recently has been focussed on the demonstration of the relationship of this protein to glutamate receptor-ion channels. In order to demonstrate such a relationship, we have taken the proteins isolated from an initial step in our purification procedure, i.e., the affinity chromatography on glutamate derivatized glass fibers and have proceeded to reconstitute these preparations in liposomes. Once the general characteristics of isolation and reconstitution were developed and the general properties of ion flux into these liposomes were determined, then studies were initiated to characterize glutamate-activated sodium ion flux into these liposomes. The end result of these studies was that glutamate was shown to activate a very rapid influx of sodium-ions into the liposomes above the level of ion flux produced in the absence of any glutamate additions. Using the lipid permeable probe sodium thiocyanate, we were able to demonstrate that the entry of sodium into the liposomes creates a transient membrane potential. The movement of thiocyanate can be used to trace the development of this membrane potential and we were able to demonstrate that not only glutamate, but also quisqualic acid and other analogs of glutamate could activate this ion flux and associated change in membrane potential. In addition known physiologic inhibitors of glutamate receptors could completely block or eliminate this ion flux and membrane potential development. On the basis of these observations we have developed a new stop-flow or quench technique and we have capitalized on this quenching procedure to develop a rapid kinetics approach to the measurement of ion flux. Following the successful demonstration of these

ion flux properties activated by glutamate and its analogs in reconstituted liposomes, we proceeded to obtain direct electrical measurements of ion channel opening and closing events in the reconstituted preparations.

In these studies the ion channel events were detected by the patch clamp procedure and by reconstitution of the proteins in planar lipid membrane bilayers. We have developed the technique of patch clamp detection and measurement of single ion channel events from reconstituted liposomes. These procedures are performed through the use of the double-dip approach through the air-water interface monolayer of lipids and protein in these reconstituted liposome preparations. The results obtained from such reconstitution studies by the patch clamp technique are shown in Fig. 1 below. As can be seen in this



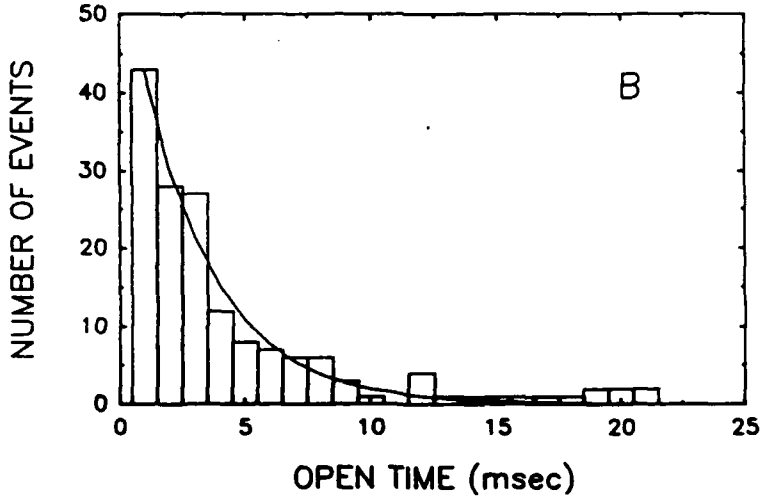


Fig. 1 Patch clamp recordings of glutamate-activated ion channels from isolated and liposome-reconstituted into PE/cholesterol liposomes. Recordings were obtained following gigaohm seal formation by the double dip technique. (A) Records of ion channel activity determined from three different reconstitution experiments and with three different concentrations of L-glutamate in the patch pipette. In the top tracing the glutamate concentration was 50 uM, in the middle tracing the concentration 250 uM, and in the two bottom tracings the concentration was 500 uM. Application of glutamate was always through the pipette solution. The applied potential for all recordings was 80 mV. Data were digitized and stored in a microcomputer and subjected to further analysis. (B) Frequency distribution of open times for the channel activity recording with 500 uM L-glutamate activation shown in (A). The mean open time duration was 7.5 msec based on analysis of 161 such events. The frequency distribution was analyzed and fit to a single exponential function (curve shown) and the calculated time constant was 2.8 msec.

figure, concentrations of glutamate from 50 to 150 mM produced activation of opening and closing events of single ion channels. The conductance of these ion channels matches very closely physiological conductance levels that have been determined in intact neuronal preparations by other investigators. In addition, the kinetic characteristics of the opening of the ion channels activated by glutamate in our preparations are almost identical to those that have been reported in the literature for glutamate receptor-ion channels and intact nerve cells. Examination of the reconstituted proteins that produced these responses indicated that the 71,000 molecular weight protein we had previously identified as the GBP is a component of three proteins that appear to form the majority of proteins in these reconstituted preparations. Therefore we believe that the protein we had previously isolated and identified as a GBP may be indeed a component of a glutamate receptor-ion channel.

In the final series of experiments performed during the contract period we have proceeded to develop the planar lipid bilayer methodology for the measurement of multiple ion channel events from reconstituted glutamate receptor-ion channel preparations. These measurements were initiated during the latter part of this contract period and led to evidence for a successful reconstitution of glutamate receptor-ion channels in these lipid bilayer membranes and a direct activation of these channels by glutamic acid at varying concentrations. A relationship between glutamate concentration and total current produced was an indication that such reconstitution could be used in the formation of a biosensor-type system that could be used to analyze low concentration of glutamic acid. These procedures are currently being pursued as a continuation of our effort to define the molecular nature and activity of glutamate receptor-ion channels.